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Germline splicing mutations of *CDKN2A* predispose to melanoma

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Coding mutations of the *CDKN2A* gene on chromosome *9p21* cosegregate with 25–60% of familial melanoma cases, but there remains a number of *9p21*-linked kindreds that lack germline coding mutations of *CDKN2A*. We sequenced *CDKN2A* exons 1α, 2, 3, and the adjacent intronic regions in 167 melanoma-prone families (at least two affected first-degree relatives), and detected four splice site variations, three of which cosegregate with the disease. RT–PCR experiments verified that these three variants, including an AGgt to ATgt mutation that demonstrates a founder effect, do affect splicing. While an exon 1α splice donor site mutation incompletely abolishes splicing, the correctly spliced mRNA yields a protein (Q50P) that cannot effectively interact with CDK4. We also performed RT–PCR on mRNA from 16 melanoma-prone kindreds to search for cryptic splice sites deep within introns, but identified no splice variants. Meanwhile, we screened 139 affected families using allele-specific PCR for the recently discovered IVS2−105A>G mutation, but found only one family that possesses this alteration. We conclude that splice site mutations do predispose to disease in a subset of melanoma-prone kindreds. Characterization of additional splice site variants and other noncoding alterations of *CDKN2A* should allow us to detect a wider range of mutations in at-risk patients.
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Introduction

Cutaneous malignant melanoma (MIM #155601) comprises about 1% of all cancer cases in European populations, but its mortality rate is over 2%, due to the propensity of this tumor to metastasize. In 8–12% of all cases, there is a family history of the disease; and in 50–80% of melanoma-prone kindreds, the disease segregates as an autosomal dominant trait linked to the *MLM2* locus on chromosome *9p21*. In addition, affected members or obligate carriers may also develop pancreatic cancer, albeit at a lower frequency than melanoma.

The tumor suppressor gene *CDKN2A* (encoding $p16^{INK4a}$) is allelic to *MLM2*, and germline coding mutations in exons 1α and 2 of *CDKN2A* cosegregate with melanoma in a subset of *9p21*-linked families. The molecular lesions that predispose to melanoma in 'mutation-negative' *9p21*-linked kindreds are unknown. We and others have sequenced the nearby *CDKN2B* gene (encoding $p15^{INK4B}$), but have observed no germline mutations of this candidate gene in melanoma-bearing individuals (Liu *et al*., 1997). Mutations of exon 1β, which encodes the alternate reading frame protein $p14^{ARF}$, do occur in cases of melanoma, but such alterations are very rare (Rizos *et al*., 2001; Hewitt *et al*., 2002). We have speculated that mutations in the noncoding regions of *CDKN2A* may be underestimated by current screening protocols. In support of this notion, we have described a G-34T transversion in the 5′ untranslated region (5′UTR) of this gene (Liu *et al*., 1999) that generates an aberrant AUG initiation codon. The mutation segregates with melanoma, and probably arose from a common founder in the United Kingdom (Liu *et al*., 1999).

We hypothesized that some noncoding mutations affect splicing of the $p16^{INK4a}$ transcript, thereby yielding gene products with reduced or absent CDK4-inhibitory function. In support of this hypothesis, Petronzelli *et al*. (2001) reported a germline G to C transversion at the splice acceptor of intron 1 (IVS1−1G>C) that segregates with affected cases in a melanoma/neurofibroma kindred. The resultant mis-spliced transcripts for $p16^{INK4a}$ and $p14^{ARF}$ both lacked exon 2. In addition,

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Harland *et al*. (2001) described an intronic mutation (IVS2−105A>G) that creates a false splice donor site, resulting in nonfunctional splice variants.

In this paper, we report additional germline *CDKN2A* splice site mutations that alter $p16^{INK4a}$ transcripts in a subset of melanoma-prone families. Specifically, by sequencing exons and the adjacent intronic regions, we found four splice site variations. RT–PCR and sequencing demonstrate that three of these affect splicing. In addition, we used RT–PCR and sequencing to search for differentially spliced products that result from cryptic splice sites deep within introns.

Results

Splice site mutations in consensus regions

Germline coding mutations of *CDKN2A* represent only a subset of melanoma-prone families that are linked to *9p21*. To address the possibility that individuals with mutations that affect splicing comprise a portion of these apparently *CDKN2A* mutation-negative families, we sequenced 20–100 bp of intronic sequences adjacent to the 5′ and 3′ regions of exons 1α and 2 to detect alterations in the splice donor and acceptor. We found four variations that can potentially affect the splicing of the $p16^{INK4A}$ mRNA (Table 1). They include, respectively, an exon 1 splice donor mutation (**A**Ggt to **C**Cgt (c. 149A>C)), an intron 1 splice acceptor polymorphism (tg**g**cagGT to tg**c**cagGT (IVS1−4G>C)); and two alterations that affect the exon 2 splice donor (AG**g**t to AG**t**t (IVS2+1G>T) and A**G**gt to A**T**gt (c. 457G>T)]. While the phenotypes and pedigrees for some of the families bearing the mutations have been described (Table 1 and references therein), we now present the molecular analyses. RT–PCR shows that the intron 1 polymorphism does not affect splicing of the message, and we will not discuss it further here.

Exon 2 mutations disrupt normal splicing

To verify that each variation alters splicing, we carried out RT–PCR on mRNA from the corresponding lymphoblastoid cell lines. Since the intron +1 G at the eukaryotic splice donor site is 100% conserved (Shapiro and Senapathy, 1987), we expected the AG**g**t to AG**t**t (IVS2+1G>T) variant to eliminate splicing activity at that site. Indeed, RT–PCR of RNA extracted from immortalized lymphoblastoid cells derived from the heterozygote mutation carrier yielded two products: one wild-type sized and another smaller mis-spliced species (Figure 1a). Interestingly, while the exon 2 G (G457) is conserved in only 78% of donor sites (Shapiro and Senapathy, 1987), the A**G**gt to A**T**gt (c. 457G>T) mutation demonstrated a pattern of mis-splicing that was identical to its AG**t**t counterpart described above (Figure 1a). We sequenced the cDNA in each case and determined that the aberrant RT–PCR products arise from the splicing of a cryptic donor site located within exon 2 to the wild-type acceptor site of exon 3 (Table 1; Figure 1b), thus splicing out 74 bp encoded by exon 2. The predicted protein products of both exon 2 donor splice mutants thus lack 24 amino acids encoded by exon 2 and possess a frame shift in exon 3 that yields six amino acids followed by a termination codon. The resultant protein lacks two of the four ankyrin repeats and – based on the structural analyses of $p16^{INK4a}$ and its relatives (Luh *et al*., 1997; Byeon *et al*., 1998; Li *et al*., 1999) – will not bind CDK4. A second laboratory has confirmed the mis-splicing of the IVS2+1 mutant (AG**t**t; Rutter *et al*., *Oncogene*, in press).

*Genotypes of families with the exon 2 A**T**gt mutation*

Among the patient samples that we analysed, individuals from two families bear the exon 2 A**T**gt mutation. This scenario may arise in two ways: (1) the mutation originated in one individual many generations ago, and apparently unrelated families bearing this mutation are in fact descended from this original founder, or (2) the alteration occurred at a mutation hotspot in *CDKN2A* in multiple founders.

To address these possibilities, we determined whether a common haplotype is shared by mutation carriers in the two families. We used polymorphic microsatellite markers to type the *CDKN2A* genomic region

Table 1 Mutations confirmed by RT–PCR to affect splicing of the *CDKN2A* mRNA

Mutation	*AGgt→AGtt*		*AGgt→ATgt*		*AGgt→CGgt*	
HGVS notation[a]	IVS2+1G>T		c. 457G>T		c. 149A>C or Q50P	
References	Hussussian *et al*. (1994) Mackie *et al*. (1998)		Moskaluk *et al*. (1998) Lynch *et al*. (2002)		Lynch *et al*. (2002)	
Family/individuals	FF	0233-212 01569-208	3679[b]	6697 6686 6726	4518[b]	6493
			1299	58 60 67		
Nationality/background	American		American		American	
Location of new splice sites[a]	Splice donation after within exon 2 (after c. 383)				Original donor site as well as within exon 1 (after c. 81)	
Resultant protein	Lacks 24 amino acids encoded by exon 2 and suffers a frame shift in exon 3				Gln50Pro missense mutation or lacks 23 amino acids encoded by exon 1, no frameshift	

[a]Human Genome Variation Society Notation http://www.genomic.unimelb.edu.au/mdi/mutnomen/examplesDNA.html. [b]The pedigrees of these families have been described in Lynch *et al*. (2002)

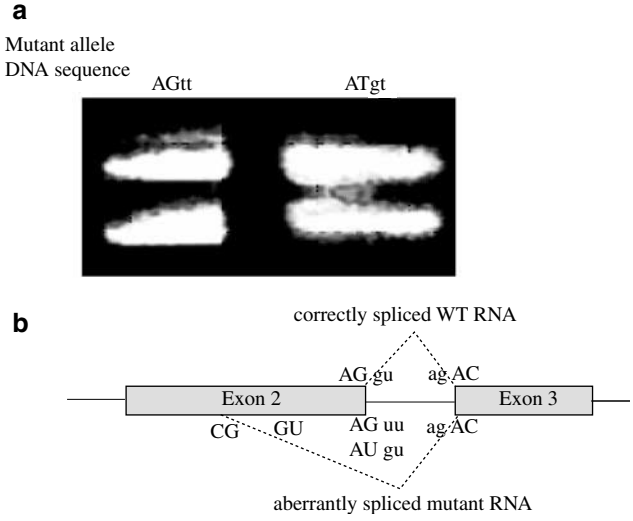
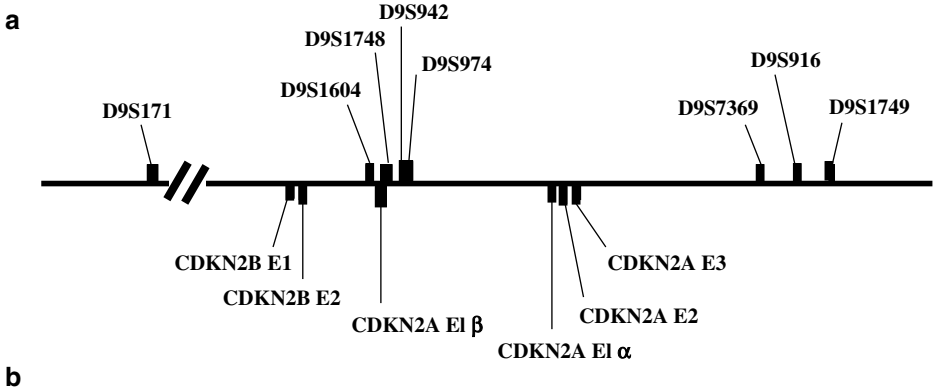


Figure 1 Splicing of mRNA in patients heterozygous for the exon 2 splice donor site mutations AGtt and ATgt. (**a**) RT–PCR products from lymphoblastoid cell lines derived from the patients with mutations. In each case, the top band represents the wild-type product (468 bp), and the bottom band is the smaller, mis-spliced version (396 bp). (**b**) Schematic representation of splice donation from exon 2 in wild-type and mutant mRNAs. Proper splicing occurs when the donor site is AGgu. When it is mutated into AGuu or AUgu, splicing occurs at the cryptic splice site CGGU within exon 2

(Figure 2a), and found that all mutation carriers share a common haplotype (Figure 2b) across eight adjacent markers spanning 2.7 Mb. Even in the case of Family 3679, where we could not determine the segregating allele for a specific microsatellite marker, the individuals still demonstrate a genotype consistent with the common haplotype. We conclude that the two families arose from a common founder bearing the A**T**gt mutation. Currently, we are unable to determine whether the family mentioned by Moskaluk *et al*. (1998) also arose from the same founder, and whether the families described by Hussussian *et al*. (1994) and MacKie *et al*. (1998) share a common haplotype with the FF family described here.

Exon 1 mutation disrupts either splicing or p16^{INK4a} function

The exon 1 mutation A149C (**A**Ggt to **C**Ggt) alters an A residue that is only 58% conserved among donor splice consensus sequences (Shapiro and Senapathy, 1987). We carried out RT–PCR of RNA extracted from the corresponding lymphoblastoid cells and detected two distinct products (Figure 3a). We first sequenced the aberrantly sized band, and observed an abnormal cDNA in which a cryptic exon 1 splice donor G81 (immediately following codon 27) is spliced to the



b

Family		3679						1299					
Individual		6697		6686		6726		126		128		130	
Marker	D9S1749	NA	NA	NA	NA	NA	NA	149	141	149	123	149	147
	D9S916	235	235	235	235	235	235	235	241	235	229	235	235
	D9S7369	157	157	157	163	157	157	157	157	157	163	157	163
	D9S974	215	221	215	221	215	205	215	209	215	211	215	211
	D9S942	100	100	100	100	100	120	100	122	100	118	100	130
	D9S1748	115	117	115	117	115	110	115	119	115	113	115	115
	D9S1604	192	192	192	192	192	194	192	192	192	192	192	194
	D9S171	173	167	173	167	173	167	173	159	173	175	173	167

Figure 2 Genotype analysis of families with the ATgt mutation. (**a**) Schematic representation of the *9p21* microsatellite markers used in the current study. (**b**) Haplotype shared by mutation-bearing individuals from the two families. The numbers in the shaded boxes indicate the haplotype shared among individuals with the ATgt mutation. Note that for Family 3679, the phase of the *D9S171* alleles could not be determined. NA = not amplified

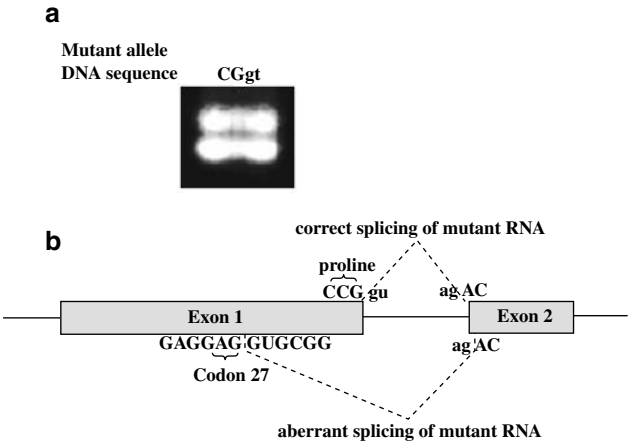


Figure 3 Splicing of mRNA in the patient heterozygous for the exon 1 splice donor site mutation CGgt. (**a**) RT–PCR products from lymphoblastoid cell lines derived from the patient with mutation. The top band represents the product with the expected size (468 bp), and the bottom band is the smaller, mis-spliced version (402 bp). (**b**) Schematic representation of splice donation from exon 1 on the mRNA expressed from the mutant allele. Proper splicing still occurs at the regular donor site, but still leads to an amino-acid change from glutamine to proline; and aberrant splicing occurs immediately after codon 27

normal exon 2 acceptor (Figure 3b). The mis-spliced mRNA lacks 69 bp encoded by exon 1, and the predicted protein suffers an in-frame deletion of 23 amino acids, which constitute part of ankyrin repeats 1 and 2. Since the ankyrin repeats are essential for the interaction between $p16^{INK4A}$ and CDK4, the mutant protein will not function as a cell cycle transition inhibitor (Byeon *et al*., 1998).

We then sequenced the normally migrating RT–PCR product and observed two cDNA species that represent transcripts from either the wild-type (A149) or the mutant (C149) allele. We conclude that the mRNA in the heterozygous lymphoblastoid cells comprises three transcripts. The A149 allele expresses wild-type mRNA, while its mutant counterpart expresses two species of message: (1) correctly spliced mRNA that predicts a missense, highly nonconservative mutation, glutamine to proline (Q50P); and (2) an aberrantly spliced mRNA that predicts a truncated and nonfunctional $p16^{INK4A}$.

The level of the aberrantly spliced mRNA species appears to be slightly higher than that of its correctly spliced counterpart, based on the intensity of the upper and lower RT–PCR bands (Figure 3a). To address this observation in a more quantitative fashion, we cloned the upper and lower PCR products into the TA cloning vector (Invitrogen) and observed 54 *vs* 47 recombinant bacterial colonies, respectively. Therefore, the ratio of upper to lower cDNA is approximately equal.

To determine the expression ratio of the wild-type *vs* missense mRNAs (both comprise the upper band), we took advantage of the fact that the A to C mutation abolishes a *Bst*NI restriction site. cDNA from the mutant C allele yields a 300 bp band upon digestion, in place of the 178 and 122 bp bands from the wild-type A allele. A total of 50 inserts were not digested by the enzyme, whereas four inserts were. Assuming a binomial distribution of wild-type (W) and mutant (M) upper and lower strands post-PCR, then the distribution of W to M is: $W^2+2WM+M^2=1$. Since the M homozygote and the WM heterozygote dsDNA is not cut with *Bst*NI, then $W^2=4$, and $2WM+M^2=50$. We calculate that the ratio of M to W (i.e. the C to A allelic ratio) is 5.3 : 2 (i.e. the steady-state level of the mutant mRNA is $\sim$2.5 times that of its wild-type counterpart in the lymphoblastoid cell line). We were unable to obtain peripheral blood lymphocytes to determine whether the proportions among wild type, mutant (correctly spliced), and mutant (mis-spliced) are similar *in vivo*.

Yeast two-hybrid analysis of the Q50P mutant protein

Although a portion of the mRNA representing the **A**Ggt to **C**Cgt mutant allele is spliced correctly, the A→C transversion does predict a highly nonconservative codon change of glutamine to proline (Q50P). To examine the function of the mutant $p16^{INK4A}$, we performed a yeast two-hybrid assay to compare the relative interaction of the Q50P mutant and its wild-type counterpart with CDK4. We used a mutant $p16^{INK4A}$ that lacks two amino acids (L104 and D105) as a negative control, since this protein has a greatly reduced level of binding activity compared to wild type at both 30 and 37°C. We fused the corresponding cDNAs to the activation domain of VP16, and the wild-type CDK4 to the DNA-binding domain of the lexA bacterial transcription factor, which specifically binds sequences within the lexA binding site upstream of the β-galactosidase gene. Interaction between functional $p16^{INK4a}$ and CDK4 results in the expression of the β-galactosidase reporter, whose enzymatic activity we quantitated in a colorimetric assay. This assay will detect temperature-sensitive mutation if performed on yeast grown both at 30°C and at 37°C; many missense variants of $p16^{INK4A}$ do exhibit this behavior (Monzon *et al*., 1998; Ruas and Peters, 1998). For each temperature, we normalize the wild-type protein as having 100% binding activity. As predicted (see Discussion), the Q50P mutant was temperature-sensitive and bound poorly to its target: we observed 8.37% of binding activity at 30°C, and only 1.37% at 37°C (Figure 4).

RT–PCR screen for splice site mutations

Direct sequencing of exons and adjacent sequences will not detect deep intronic mutations that affect splicing. To identify novel transcripts resulting from aberrant splicing events due to such alterations, we extracted RNA from EBV-transformed lymphoblastoid cells of 18 cases derived from 16 melanoma-prone families, 15 of which lacked germline coding or splice site mutations of *CDKN2A*. As control, we used RNA from lymphoblastoid cells from a member of the 16th family. This patient bears a 19-bp deletion in exon 2, which results in a novel

mRNA 19 bases shorter than its wild-type counterpart (Gruis *et al*., 1995).

We performed first-strand synthesis using a specific primer to sequences in the 3′UTR (5′ end at 176 bp after the stop codon) and amplified the cDNA by PCR using primers PCR1-f and PCR1-r (Table 2). We amplified an aliquot of the first PCR with nested primers PCR2-fa and PCR2-r (Table 2) and analysed the products by agarose gel electrophoresis. We did not observe differentially sized alleles, as might be expected from aberrant splicing. Since agarose gels do not resolve DNA species with slight differences in length, we also sequenced each RT–PCR product to search for ambiguous sequences that arose from mis-splicing events. We found that all RT–PCR products represented wild-type cDNA species (data not shown).

Screening of the IVS2−105A>G mutation

Recently, Harland *et al*. (2001) reported an intronic mutation of *CDKN2A* in six English melanoma pedigrees (IVS2−105A>G) that creates a novel splice donor site 105 bases 5′ of exon 3. Using allele-specific PCR, we screened 139 melanoma-prone families (ascertained up to September 2001) that lack known melanoma-associated mutations. We found only one family that bears the variant, and conclude that the prevalence of this mutation in the multiethnic Toronto population is less than its prevalence in the UK population sampled by Harland *et al*. (2001).

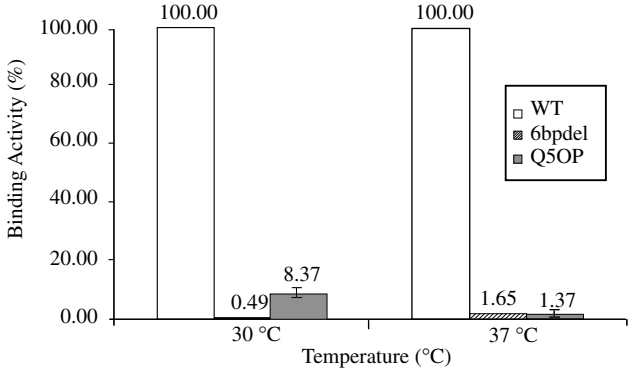


Figure 4 Comparison of protein binding activities using the yeast two-hybrid system at 30 and 37°C. The binding of the $p16^{INK4a}$ proteins to CDK4 is normalized to the wild-type $p16^{INK4a}$. The binding activities of a negative control (6 bp del) and the Q50P mutant are measured

Discussion

The role of *CDKN2A* germline mutations in melanoma has been investigated by many groups worldwide. In the majority of cases, the frequency of germline coding mutations observed in the coding regions of the *CDKN2A* gene is less than expected, given the frequency of melanoma families linked to chromosome *9p21*. In addition, the frequency and specific mutation type of *CDKN2A* alterations vary enormously in different populations (Hogg *et al*., 1998). We have hypothesized that such differences in *CDKN2A* mutation detection might be explained either by (1) noncoding mutations of the gene, as explored in this study; or (2) by the effect of other melanoma susceptibility genes (Hogg *et al*., 1998). The discoveries of melanoma-predisposing mutations in the 5′UTR (Liu *et al*., 1999) and in the introns (Harland *et al*., 2001; Petronzelli *et al*., 2001) of *CDKN2A* support this hypothesis.

To address the possibility that aberrant splicing of the $p16^{INK4a}$ message might be associated with melanoma susceptibility, we screened patients from families that lacked germline *CDKN2A* mutations by a combination of two methods: first, we sequenced genomic DNA to detect mutations of the wild-type splice donor, acceptor, or branch point sites that directly alter normal splicing events. The three mutations (AG**t**t, A**T**gt, and **C**Ggt) that affect splicing belong to this category. Second, we searched for deep intronic mutations that might create novel splice donor or acceptor sites. The discovery of the intronic mutation by Harland *et al*. (2001) demonstrated that mutations that create a novel splice site can indeed occur. We did not detect such mis-splicing events by RT–PCR analysis of $p16^{INK4A}$ lymphoblastoid mRNA. However, we cannot exclude the possibility that some intronic mutations might elude detection by RT–PCR by splicing to novel 3′ sequences that are unrecognized by our current oligonucleotide primers.

Individuals in the two families that bear the exon 2 A**T**gt mutation share a common haplotype, indicating

Table 2 Primers used in PCR and sequencing analysis of *CDKN2A*

Primer	*Sequence (5′ to 3′)*	*Location of 5′ end of primer*[a]	*Direction*	*Size of PCR product (bp)*
E3PCR-f	GGGAAGGGAAAGGCCACATC	IVS2−373	Forward	575
E3PCR-r	TGGACATTTACGGTAGTGGG	c. 660	Reverse	
RT	TTACGGTAGTGGGGGAAGGC	c. 653	Reverse	N/A
PCR1-f	AAAGAGGAGGGGCTGGCTGGTCAC	c. −100	Forward	660
PCR1-r	GTTGTGGCGGGGGCAGTTGTGGC	c. 560	Reverse	
PCR2-fa	CCGGGGTCGGGTAGAGGAG	c. 62	Forward	468
PCR2-r	GACCTTCGGTGACTGATGAT	c. 530	Reverse	
PCR2-fb	AGAACAGACAACGGGCGGC	c. −30	Forward	548
PCR2-r	(See information above on PCR2-r)			

[a]Human Genome Variation Society Notation http://www.genomic.unimelb.edu.au/mdi/mutnomen/examplesDNA.html

this mutation arose in a founder common to both kindreds. Founder effects occur in many *CDKN2A* mutations, including: G101W (Ciotti *et al*., 2000), an exon 2 19-bp deletion (Gruis *et al*., 1995), 113 ins R (Hashemi *et al*., 2001), M53I (Pollock *et al*., 1998), G-34T (Liu *et al*., 1999) and V126D (Goldstein *et al*., 2001). Most of these mutations occur within specific geographical locations and/or ethnic groups. Of note, we detected the −105 intronic splice donor mutation in only one of 139 (0.7%) melanoma families that lack coding mutation of *CDKN2A*. In contrast, Harland *et al*. (2001) described this alteration in six of 84 (7%) coding mutation-negative UK families. This discrepancy in mutation detection frequency of one order of magnitude highlights the importance of defining the population(s) in which *CDKN2A* analysis is to be performed. To date, only one *CDKN2A* variant (the N-terminal 24-bp duplication) is an exception to this rule, as it arises in a mutation hotspot. Specifically, haplotype analyses at this locus have demonstrated at least five independent founders (Pollock *et al*., 1998 and our lab data). This region of *CDKN2A* is probably inherently unstable due to mitotic recombination between two imperfect 24-bp repeats naturally present in the wild-type sequence (Pollock *et al*., 1998).

It is essential to analyse mRNA products from cell lines or tissues in order to understand the functional consequences of a potential splice site mutation. For instance, Hussussian *et al*. (1994) previously predicted that the mis-splicing event stemming from the AG**g**t to AG**t**t mutation would lead to codon changes from DIPD* (exon 3) to GED* (intron 2), due to read-through past the exon 2 donor splice site. In fact, an RT–PCR assay using message from AGgt/AG**t**t heterozygous lymphoblastoid cells demonstrated a cryptic splice donor site arising in exon 2, the use of which could not have been predicted prior to our analysis. Characterization of additional splice site mutations revealed that the same cryptic site was used in the exon 2 donor mutation A**G**gt to A**T**gt.

There has been considerable debate as to whether germline mutations of *CDKN2A* are solely responsible for melanoma susceptibility, or whether mutations in exon 2 that also affect the predicted sequence of $p14^{ARF}$ play a role in tumorigenesis. Quelle *et al*. (1997) introduced six missense mutations into the shared sequences of $p16^{INK4A}$ and $p14^{ARF}$. Of these, two caused the complete loss of $p16^{INK4A}$ function, but none affected $p14^{ARF}$. While exon 1β, which encodes protein sequences unique to $p14^{ARF}$, is necessary and sufficient for G1 arrest *in vitro*, it is not clear that a similar G1 arrest will occur *in vivo* (Rizos *et al*., 2001). However, many mutations of exon 2 affect $p16^{INK4A}$ sequence and function alone, and leave $p14^{ARF}$ unchanged (Quelle *et al*., 1997). In keeping with these observations, the exon 2 cryptic splice donor site that we describe in this paper is located downstream of the $p14^{ARF}$ termination codon.

Although mRNA from the **A**Ggt to **C**Cgt mutant allele can be spliced correctly, the resultant mRNA predicts a highly nonconservative codon change of glutamine to proline (Q50P). We predicted that this alteration would adversely affect protein function for the following reasons: first, $p16^{INK4A}$ contains four helix–turn–helix structures known as ankyrin motifs, the second and third of which contribute most of the interactions between $p16^{INK4a}$ and CDK4/6 (Byeon *et al*., 1998). Q50 is part of a one-turn helix that constitutes the first portion of the second motif, and this residue stabilizes the organization of the second ankyrin repeat and its adjacent neighbors. In addition, Q50 is a component of a polar pocket that binds CDK4 (Byeon *et al*., 1998). Taken together, there is little doubt that function of the Q50P variant will be compromised. To confirm this hypothesis, we performed a yeast two-hybrid assay to compare the relative interaction of the Q50P mutant and its wild-type counterpart with CDK4. As predicted, the mutant $p16^{INK4A}$ interacted poorly with its target.

In summary, we have demonstrated that splice site mutations in the *CDKN2A* gene contribute to melanoma predisposition. Taken together with previously described mutations in the 5′UTR and both introns, these data demonstrate that noncoding mutations of this gene play a significant role in melanoma susceptibility.

Materials and methods

DNA, RNA, and cell lines

The population in our study comprises of 167 American and Canadian melanoma-prone kindreds, defined as families with at least two affected first-degree relatives, who have no known melanoma-associated mutations. Following informed consent (The Sunnybrook and Women's College Health Sciences Centre, Toronto, ON, Canada), we drew blood from affected members of melanoma-prone kindreds, prepared DNA and RNA (Liu *et al*., 1995), and transformed the lymphocytes with Epstein–Barr Virus (EBV) as previously described (Coligan *et al*., 1999). We expanded the immortalized cell lines in RPMI-1640 media with 15% heat-inactivated fetal bovine serum (56°C for 30–45 min), 2 mM L-glutamine, and 10 μg/μl gentamycin (all GIBCO BRL, MD, USA) until they reached a density of 8×10^5 cells/ml.

Germline CDKN2A *sequencing*

For all 167 samples, we sequenced exons 1α and 2 of the *CDKN2A* gene as described in Liu *et al*. (1999). In addition, we amplified exon 3 using the primers E3PCR-f and E3PCR-r and sequenced the products using the same primers (Table 2).

Confirmation and detection of splice site mutations with RT–PCR of the CDKN2A *mRNA*

We annealed 10 pmol of the reverse transcription primer (RT; Table 2) to 3 μg of total RNA at 70°C, and carried out first-strand cDNA synthesis using Superscript II Reverse Transcriptase (GIBCO BRL) at 42°C. After treatment with RNase H (MBI Fermentas, ON, Canada), we amplified the cDNA using primers PCR1-f and PCR1-r, and further amplified these products with the nested primers PCR2-fa and PCR2-r (Table 2). Both PCR reactions used the *Taq* polymerase (GIBCO BRL) with standard buffer conditions, 5% DMSO, 1.6 mM $MgCl_2$, and 0.2 mM dNTP. The PCR products were

analysed on 1.5% agarose gels and purified using the Concert™ Rapid Gel Extraction Purification System (GIBCO BRL).

Sequencing of RT–PCR products

We sequenced the RT–PCR products (30 ng) on both strands using the primers PCR2-fa and PCR2-r (Table 2). We carried out the reaction using the dRhodamine Terminator Ready Reaction Mix (ABI PRISM Dye Terminator Kit, Perkin-Elmer, CA, USA) and the accompanying protocol, and analysed the reaction mixtures using the ABI PRISM310 Genetic Analyzer (Perkin-Elmer).

*Genotype analysis for the exon 2 A**T**gt mutation*

To determine whether the occurrence of the A**T**gt mutation in the two families (Table 1) is a result of a founder effect, we performed genotype analysis on the DNA of the affected individuals who bear the variant. The microsatellite markers used in the analysis were *D9S1749, D9S916, D9S7369, D9S974, D9S942, D9S1748, D9S1604*, and *D9S171* (Figure 2a). We obtained primer sequences for all markers (except *D9S7369*) from the Genome Database (http://www.gdb.org/), and designed primers for marker *D9S7369*: forward 5′-GATACAATGCTGGATTTCGTTTGCTA-3′ and reverse 5′-GGACTCCTCTCTAACTTATTC-3′. We labeled all forward primers with one of 5-FAM, HEX, or TET (Integrated DNA Technologies, IA, USA), and typed each marker using standard PCR conditions (above) at an annealing temperature of 3°C for 35 cycles. We then ran aliquots of the PCR products on the MJ Basestation (MJ Research, CA, USA), and scored the sizes of the alleles using the computer program Cartographer v1.2.6sg (MJ Bioworks, WI, USA).

*Quantitation of splice variants of the **A**Ggt→**C**Ggt mutation*

We extracted total RNA from the lymphoblastoid cell line bearing the **A**Ggt→**C**Ggt mutation at the exon 1 donor site, and performed RT and first PCR as described above. Then we further amplified the cDNA with nested primers PCR2-fb and PCR2-r (Table 2), and analysed the products on 1.5% agarose gels. Finally, we ligated the products into the TA cloning vector and transformed Top10F′ cells using the Original TA Cloning® Kit (Invitrogen, CA, USA).

We picked colonies bearing insertions, diluted each in 10 μl of sterile water, and screened 1 μl of the diluted bacteria with PCR using primers PCR2-fb and PCR2-r. We analysed the PCR products on 1.5% agarose gels, and purified the wild-type-sized band PCR products using the Concert™ Rapid Gel Extraction Purification System. We digested the purified PCR product with *Bst*NI (New England Biolabs, ON, USA), and analysed the products on 1.5% agarose gels. The C allele yields a 300 bp band upon digestion, in place of the 178 and 122 bp bands from the wild-type A allele.

Yeast two-hybrid assay

Using PCR-based mutagenesis techniques, we engineered the Q50P mutation into a pVP16 vector (gift from S Hollenberg, Fred Hutchinson Cancer Research Center, Seattle) that already contained the full-length cDNA of *CDKN2A*. We substituted the wild-type CDKN2A cDNA in the original vector with one that contained the Q50P mutation, and performed a colorimetric yeast two-hybrid assay as previously described in Monzon *et al*. (1998) to assess the interaction between the Q50P mutant and CDK4.

Allele-specific PCR for the IVS2−105A>G mutation

We sampled at least one affected individual from 139 different melanoma families (ascertained up to September 2001) who had previously tested negative for coding p16 mutations. These 'melanoma families' have at least two members with the disease, and were ascertained between March 1996 and December 2000 through the Familial Melanoma Clinic at the Toronto-Sunnybrook Regional Cancer Centre. Using the primers WT-F, Mut-F, and p16-3R (Harland *et al*., 2001) and control primers cdk6-F and cdk6-R (Shennan *et al*., 2000), we screened these families for the IVS2−105A>G mutation (Harland *et al*., 2001). The PCR reactions, modified from Harland *et al*. (2001), used the *Taq* polymerase (GIBCO BRL) with standard buffer conditions, 1.5 mM $MgCl_2$ and 0.2 mM dNTP. We employed the following amplification conditions: 96°C – 1 min; five cycles of (96°C – 25 s, 70°C – 45 s, and 72°C – 45 s); 21 cycles of (96°C – 25 s, 65°C – 50 s, and 72°C – 45 s); 4 cycles of (96°C – 25 s, 55°C – 1 min, and 72°C – 2 min); and 72°C – 7 min. The PCR products were analysed on 1.5% agarose gels.

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